From Herbivory to Egg Counting: The Use of Digital Microscopy and Image J to Capture Images and Analyze Data for Ecology Projects

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Abstract
Ecology labs can be enhanced by the use of digital photomicroscopy and the free software program, Image J. Images of leaves that have undergone damage by herbivory by insects can be captured with a Motic digital camera and analyses such as total area of damage can be conducted using Image J. Another use of Image J is cell counting. Gravid (egg-bearing) *Palaemonetes spp.*shrimp can be collected in the wild (or collected and shipped live by Gulf Specimen Marine Lab). Eggs can be counted more accurately with the assistance of Image J, and egg counts (fecundity) can be compared across species and location. These two projects are just two of many projects that could be conducted with the assistance of this technology.

Introduction

Sometimes it is difficult to devise interesting and engaging labs for the Ecology laboratory because of limitations of field work. This exercise introduces two projects that utilize materials that are easy to collect: leaves that have undergone herbivory by insects and eggs collected from the abdomens of easily-collectable shrimp. Once the specimens have been collected, digital photomicroscopy can be used to capture images and a free software program, Image J, can be used to assist in data analysis.

Fordham University owns a research facility, the Calder Labs, which is a field station in Armonk, New York that specializes in forest ecology. Tara Glover, a St. Francis College student, participated in a National Sciences Foundation program titled "Research Experiences for Undergraduat**es**", in which she measured the area of herbivory in leaves by insects with the assistance of Image J. (She later uncovered several RNA sequences that were probably responsible for producing proteins that had an immune effect for the plants that were stimulated by the herbivory.) This was part of a comprehensive project to determine the extent of damage of plants by insects (Glover and Lewis, 2006).

Image J can also be utilized for cell counting. Upon collecting various estuarine shrimp species (*Palaemonetes spp.* and *Cragnon septemspinosa*) we discovered several gravid (egg-bearing—on the abdomen) females. We thought it would be interesting to study the fecundity of these shrimp, and record times of year when they were fecund. This was a part of an overall project in which we were attempting to determine the level of gene flow between shrimp of two New York locales. (Phoolbosseea and Nolan 2007). When searching for a suitable shrimp (*Palaemonetes spp*) egg counting technique, we discovered that placing a small amount of eggs into a small ring (used for protecting 3-hole binder pages) on a microscope slide yields a field of view that matches that visible view under the dissection scope that is appropriate for photomicroscopy and accurate counting.

To use Image J to assist in egg counting, place several rings on the slide and number them. Take a picture of each ring that has been filled with eggs, a drop of sea water, and a drop of glycerol, and save it. Open each picture in Image J, place the arrow of the mouse on each picture and click on each egg. The picture of each egg will be replaced by a white square, which will indicate that it has been counted. The count is then automatically tallied.

These are just two examples of what one can do with Image J; besides measuring the area of herbivory in a leaf, and counting eggs, one could measure the diameter of any cell, and the numbers of cells for an infinite number of studies. We have used the Motic camera and Image J to measure the diameter of amyloplasts, which are starch granules that have been isolated from peas, counted and measured the size of stomata in plants under various conditions, and counted and measured red and white blood cells. The camera and software also have the capability of taking short videotapes, which are invaluable for recording movement of protozoa collected from pond water. We have found that on the computer screen the visibility of protozoa such as Paramecia is enhanced. One can actually see the central vacuole and the cilia beating. It is also easier to detect the 3 D nature of this single-celled organism.

These techniques are appropriate for all levels; we have conducted the herbivory analysis and egg counts in our sophomore/junior level ecology course.

Time is required to set up the cameras and computers, and collect the leaves and shrimp. One three-hour laboratory period could be used to introduce the two projects and the use of the camera and Image J. These exercises could also be used for pilot projects for independent research projects.

Student Instructions

Leaf herbivory (chewing) by insects is an important ecological phenomenon. We may think of this only in terms of crop destruction, but it occurs naturally in the wild. You can easily set up research projects to determine the extent of herbivory by different insects and/or different plants. Comparative studies could easily be generated. This can be expressed as the percentage of leaf that has undergone herbivory. You may take pictures of leaves that have undergone herbivory, and then, with the use of the software called "Image J" determine area of herbivory, and then express this as a percentage of the total area.

Fig. 1

Calculating Insect Herbivory
SET UP IMAGE

1. Open Image J. Then open scanned file that contains leaf. (You may take digital pictures of leaves that contain herbivory and save them as a JPEG file.) Fig. 1. is an example.
2. Click IMAGE →ADJUST → BRIGHTNESS/CONSTRAST. A new box will open up. You want to adjust picture to configure file and eliminate the veins within the leaf which distort data.
3. The MINIMUM VALUE in the pop-up box should be increased from 0 to 74 to make the picture darker. All other values will adjust.
4. Close out BRIGHTNESS/CONSTRAST pop-up box.
5. Click on IMAGE then TYPE→ 8-Bit.
6. Next, click PROCESS → BINARY →MAKE BINARY. The image will now be in black and white. This should completely eliminate the veins within leaf. Save a copy of this image as "Total area" picture for later on.

HIGHLIGHTING AREAS OF HERBIVORY

Fig. 2

7. You will now draw in areas of herbivory. Choose PROCESS → BINARY → OUTLINE. Open original file again to compare images.
8. Click Freehand line selections (heart-shaped picture on draw tool bar just below menu choices) and outline an area of herbivory. Immediately right-click and select "FILL".
9. Repeat for all other areas of herbivory.
10. Save image as.JPEG File under a new name.
11. In your calculations, you only want areas of herbivory, so you have to erase outline of leaf. Click on Freehand selections and draw over areas, one-at-a-time, that you want to eliminate. Right-click on "CLEAR" and selected area will disappear. Click again outside of area for selection line to disappear. Continue to erase until all that are left will be the areas of herbivory.

CALCULATING AREA OF HERBIVORY
12. Click ANALYZE → SET SCALE
This will be demonstrated to you by your instructor---the scale will be different for each type of picture or microscope. For example, if your leaf is 25 cm long, and the pixel scale on your JPEG picture is 1023 pixels, then 41 pixels would equal 1 cm. You may add these values to the set scale. Note that you need to reset this scale anytime you do a new calculation.
13. Click ANALYZE → ANALYZE PARTICLES
      i.   Check: Summary
     ii.   Show: Outline
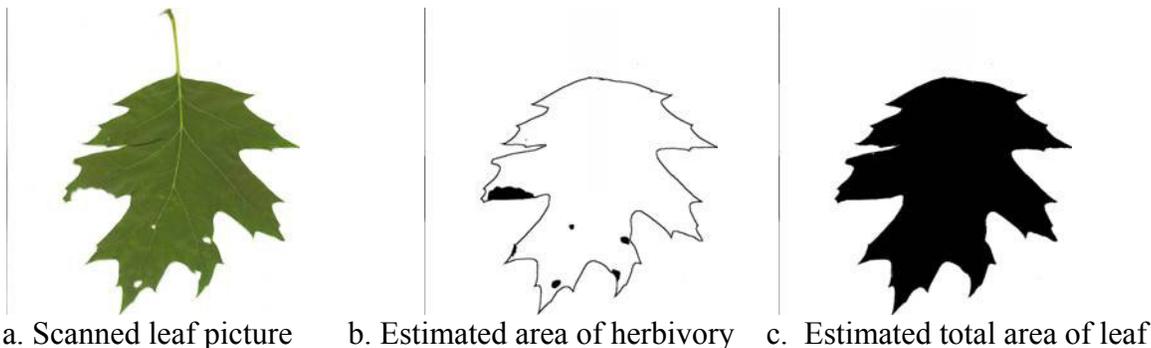   Click OK.

Fig. 3

14. Open SAVED "Total area" file from STEP. 6.
15. Using the free selction tool and right-clicking on "FILL", fill in all empty areas in black. This entire leaf should be completely filled in. Fig. 3. .
16. Follow STEP 12-13;. You now have TOTAL AREA.
17. Calculate percentage of herbivory.

     AREA OF HERBIVORY / TOTAL AREA X 100 = % of herbivory

a. Scanned leaf picture   b. Estimated area of herbivory   c. Estimated total area of leaf
Figure 4. Examples of leaf pictures for use with herbivory exercise.

Counting eggs from gravid *Palaemonetes spp.*
    Palaemonetes spp. are shrimp that are very common along the coast of the eastern Atlantic seaboard, mostly in brackish inlets just off the coast. They serve as food for fish and birds, and thus are an important part of the estuary. In this exercise you will do a comparative study of the biotic potential of these shrimp by counting the number of eggs that they are carrying. This is a measurement of their fecundity. What would be the implications of your findings?
    "Gravid", in this case refers to the shrimp that are "carrying eggs" on their abdomen (L. *gravidus*, from gravis "burdened, heavy").

1. Examine a gravid shrimp under the dissection scope. Which species do you have, *Palaemonetes pugio, P. vulgaris* or *P. intermedius*? Use the field guide (Gosner, 1999) to assist you in your identification.
2. Where was it collected? How many eggs do you think your specimen contains?

3. Attach the Motic camera to the dissecting scope and to the computer. Capture an image of the rostrum of the shrimp (now you can see why they are sometimes called the "dagger-blade" shrimp. Save the picture for your report.

4. Put several reinforcement rings (normally used for three-hole paper) on a microscope slide. Number the rings with a marking pen. Place a drop of sea water and a drop of glycerol into the center of each one.

5. Next, place a small amount of eggs into each ring and spread them apart so that they make a monolayer. (This is difficult to do---you can place the whole egg mass on a separate slide and tease apart the eggs with two pins.) Why do you think you are required to place the eggs in sea water instead of distilled or tap water?

6. The area formed by the space in the ring should be within your field of view when you look through the oculars of the dissecting scope. Capture an image of each ring of eggs and save as a jpeg (each picture will be labeled "shrimp 1, shrimp 2, etc.) Return the eggs to a separate microfuge tube that you have first filled halfway with glycerol. What is the purpose of the glycerol? In this way you can save the eggs for further study.

7. Open Image J. Then open the first shrimp picture. Image J will be visible as a smaller rectangle on the screen.

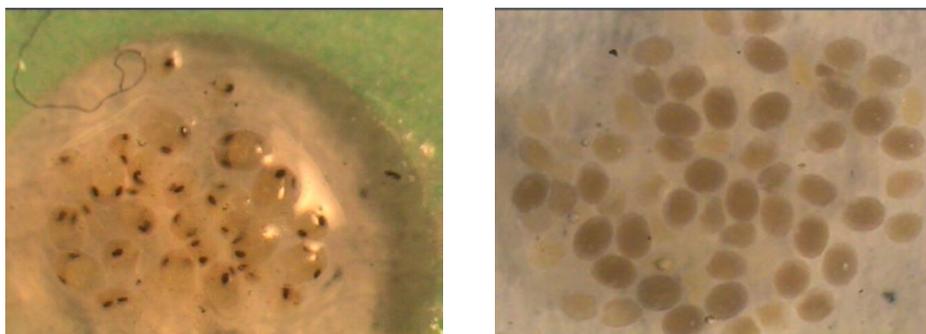8. Go to "Plugins" and drop down to "Analyze", and over to "Cell Counter".

Figure 5. Sample picture of shrimp eggs

9. A box will open in which you will be asked to define a "particle" which you will be counting. Define one particle type. Click on one egg while using the mouse with the arrow. A dot should appear in the egg. Continue clicking on all the eggs in your field of view. A dot will be placed in each one, and a tally will be given. Write down the total.

10. Close the picture, and repeat with the rest of the shrimp pictures until you have finished counting all the eggs. Total your totals for each picture.

This way of counting will allow you to "lose track" of where you left off counting, in case you are interrupted. The "dot" placed on each egg will assure that you won't count that egg twice. This method is probably more accurate than other methods---what do you think? Save a few pictures of the eggs to insert into your lab report. Do they all look alike?

(Optional)
Image J Analysis of Organelle Length using Amyloplasts as a model
1. Take peas that have been soaking in a beaker (100 g of peas soaking in 500 ml of water for several hours or overnight should be sufficient for the whole class).
2. Blend the peas in a blender.
3. Pour through two layers of cheesecloth.
4. Take a very small speck of material from the cheesecloth, place it on a microscope slide, add a drop of Lugols or tincture of iodine.
5. Place a coverglass on top and flatten the speck out with gentle pressure from your thumb or a pencil eraser.
6. Examine under low power for tiny black dots—these are the amyloplasts, or organelles that contain amylase. What type of molecule is amylase, and how do we know this?
7. Capture an image of the amyloplasts with the Motic Camera. Using Image J, what is the total area of the amyloplast? Can you estimate the volume?
8. File- Open
9. Open Saved .jpeg file of your choice (or Figure 3.)
10. Choose Image-Type-"8-Bit" (8 bit means the amount of color, gray scale)
11. Click Process- Binary- Threshold
12. Click Analyze- Set Scale
13. Highlight image using Free hand selections. (trace image)
14. Click Analyze – Analyze Particles
    a. Show: Outline
    b. Check: Display Results And Summarize
15. Click OK.

What other plants could you isolate plastids from? What are the implications of number and size of plastids as to the nutritional content of various plants?

Figure 6. Amyloplasts

Materials

Motic camera and software (Motic Images Plus 2.0 ML)
(available from Carolina Biological, 2700 York Rd., Burlington, N.C. (800-334-5551) or
Micro-Optics, 68-23 Fresh Meadows Lane, Fresh Meadows, NY, 11365)
Image J (free downloadable software from NIH) Also download Cell Counter plug-in.
Leaves that exhibit herbivory (collected ahead of time by instructor, or by students on a field trip---scanned pictures or digital files of leaves, may also be provided)
Gravid shrimp can be purchased from Gulf Specimen Marine Lab P.O. Box 237, Panacea, FL 32346
Phone: (850) 984-5297
Glycerol in dropping bottles
Reinforcement rings (for 3 hole-paper)
Sharpies
Pond water or protozoan cultures
Pipettes
Microscope slides
Proto-slo or 3% methyl cellulose
Peas
Blender
Beaker
cheesecloth
Cover glasses
Lugols reagent

Notes for the instructor

Each microscope will require a certain sized adapter----order at least two, one for your dissecting and the other for your compound microscopes. Micro-optics sells these. At many schools, including ours, we need to have an administrator load all software, including the Motic and the Image J. The camera must be plugged in and recognized by the software; for us, an administrative password was required also.

Acknowledgments:

Literature Cited

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About the Authors:

Tara Glover, Islam Aly, Franchette Viloria and Eugene DiDonato were St. Francis College undergraduates who assisted in collecting materials and developing the project.

Tara Glover conceived of and wrote much of the section on insect herbivory as part of a National Science Foundation Research Experiences for Undergraduates fellowship she received from Fordham University. Tara Glover is now pursuing a Master's degree in Science Education from Long Island University.
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Todd Nickle has been a biology educator at what was once known as Mount Royal College (but is now a university)for the past 10 years. He is interested in active teaching and learning techniques and credits ABLE for much of the innovation that has been incorporated into the seminal years of young Calgary biologists. His contribution to this paper was to clarify and field-test some of the instructions for image capture and analysis.
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Allen Burdowski, Ph.D. in Biology, is the Associate Dean and a Professor in the Department of Biology and Health Promotion at St Francis College. He conceived of the idea of counting the shrimp eggs using Image J, and provided training for the students and faculty in using the Motic camera and Image J. He has taught many biology courses over the years at Yeshiva University, New York University and St. Francis College, including physiology, biology, medical informatics, pharmacology and histology.
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Lynda Valanzano graduated as a biology major and is now a lab instructor at St. Francis College. She teaches Anatomy and Physiology and General Biology labs, and works on research projects with students.
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Kathleen Nolan, Ph.D. is the current Chair of Biology and Health Promotion, and has been a member of ABLE for many years. She has presented several major and mini-workshops at ABLE and is very interested in estuarine education, and lab education in general.
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Appendix

Motic camera can be purchased from:
Carolina Biological, 2700 York Rd., Burlington, N.C. (800-334-5551) or
Micro-Optics, 68-23 Fresh Meadows Lane, Fresh Meadows, NY, 11365)

Frozen gravid shrimp (or any fish egg samples) can be purchased from Gulf Specimen Marine Lab P.O. Box 237, Panacea, FL 32346
Phone: (850) 984-5297